REMARKS

Prior to the present amendment, claims 1-14 and 30 were pending and claims 15-29 and 31 were withdrawn by the examiner as being directed to non-elected inventions. By this amendment, claims 1-6, 8, 11-12 and 30 have been amended and new claims 32-36 have been added. Accordingly, claims 1-14, 30 and 32-36 are currently pending.

In the Office Action, claims 1-14 and 30 were rejected under 35 U.S.C. §112, second paragraph for allegedly being indefinite for various reasons which are addressed below.

The examiner states that applicant is required to comply with the sequence rules by inserting the sequence identification numbers of all sequences recited within the claims and specification. Applicant has complied with the sequence rules and amended the claims and specification by inserting the sequence identification numbers of all sequences recited within the specification and claims.

Claims 1-14 and 30 were rejected under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claims 1-14 and 23 of U.S. Patent No. 5,928,928.

The Examiner indicates that the filing of Terminal Disclaimers may be used to overcome this rejection. Accordingly, a Terminal Disclaimer in compliance with 37 CRF §1.321(c) is submitted herewith. It is respectfully submitted that this Terminal Disclaimer is in proper form and overcomes the double patenting rejection.

The examiner alleges that claims 1-4 are indefinite for the recitation of the phrase "essentially corresponding to." According to the examiner, it is unclear how many changes can be made within the recited sequence and still be included within this phrase. The examiner acknowledges that page 12 of the specification teaches that generally the changes will be less than 30% of the total number of amino acids or nucleotides in the recited sequence.

Applicant has amended claim 1 to recite " an essentially corresponding form of said human chitinase having a sequence homology of at least 70%, having chitin-hydrolyzing activity." Accordingly, the claim now recites the maximum number of changes which may be made within the recited sequence.

According to the examiner, claim 1 is indefinite in the recitation of "modified form of said human chitinase having a substantially similar chitin-hydrolyzing activity." In response, applicant has replaced the phrase "modified form of said human chitinase having a substantially similar chitin-hydrolyzing activity" with the phrase "an essentially corresponding form of said human chitinase having a sequence homology of at least 70%, having chitin-hydrolyzing activity."

The examiner further contends that claim 11 is indefinite for reciting "cosmetic (e.g. body lotion)," dental (e.g. tooth paste, month rinse)," and "food product (e.g. milk, cheese and other dairy products)." Applicant has amended claim 11 by deleting the various phrases "(e.g. ...)" from the claim. In addition, new dependent claims 32-36 have been added to recite the exemplifications recited in claim 11.

Claim 30 was rejected allegedly for indefiniteness for reciting "a conventional component of diagnostic kits for detecting an antigen or antibody." The examiner asserts that it is unclear what things would be considered "conventional components."

Applicant has amended claim 30 be replacing the phrase "a conventional component of diagnostic kits for detecting an antigen or antibody" with the phrase "human chitinase antigenic peptide, a human chitinase antibody, a recombinant human chitinase nucleic acid or a human chitinase oligonucleotide." Support for the amendment to the claim can be found in the specification as originally filed, *inter alia*, page 16, lines 4-12.

In view of the above amendments and remarks, applicant respectfully requests that the rejection of claims 1-14 and 30 under 35 U.S.C. §112, second paragraph be reconsidered and withdrawn.

Claims 1, 4-14 and 30 were rejected under 35 U.S.C. §112, first paragraph for alleged lack of written description. The examiner states that these claims are directed to polypeptides corresponding to SEQ ID NO: 4 and 6 and variants thereof having substantially similar chitin-hydrolyzing activity. According to the examiner, no description has been provided of the modified polypeptide sequences encompassed by the claim. The examiner concludes that, in view of the lack of description of representative species encompassed by the genus of the claim, the specification fails to sufficiently describe the claimed invention such that a skilled artisan would recognize that applicants were in possession of the claimed invention.

Applicant respectfully disagree. The specification discloses a variant having SEQ ID NO:6. This sequence has 82% homology with the sequence of SEQ ID NO:4. Moreover, the specification gives adequate guidance for the generation of further variants having chitinase activity.

For example, the entire region specified by exon F is not essential for chitinase activity (see *inter alia,* fig. 3 and SEQ ID NO: 6 of the specification as originally filed). Therefore, this region may thus be modified according to the specification to yield variants. Moreover, the specification discloses regions that can be modified without essentially affecting chitinase activity. For example, page 30, lines 6-17 discloses several C-terminal modifications which yield active chitinases.

In addition, the specification provides details as to critical sites, in which modifications are not likely to be tolerated. Such critical sites include, for example, the highly conserved region presumed to be the catalytic center region of chitinase (see *inter alia,* page 20, lines 23-27). Further, page 30, lines 23-29 of the specification discloses additional regions of conservation. A person skilled in the art knows that modifications to the highly conserved regions should be limited to the modifications that are found in other chitinases (see figure 4).

Therefore, the claimed invention is adequately described in the specification. Accordingly, applicant respectfully requests that the rejection of claims 1, 5-14 and 30 under 35 U.S.C. §112, first paragraph be reconsidered and withdrawn

Claims 1-14 and 30 were rejected under 35 U.S.C. §112, first paragraph. The examiner acknowledges that the specification is enabled for human chitinases of SEQ ID NO: 4 or 6 or chitinases encoded by nucleic acids which hybridize to the nucleic acids of SEQ ID NOs: 3 or 5 under specific stringent conditions. However, the examiner contends that the claim is not enabled for any chitinase "essentially corresponding " to SEQ ID NO:4 or 6 or "having substantially similar chitin-hydrolyzing activity."

As stated above, applicants have amended the claims to recite "a human chitinase having an amino acid sequence selected from the group consisting of an amino acid sequence depicted in SEQ ID NO:4, or an essentially corresponding form of said human chitinase having a sequence homology of at least 70%, having chitin-hydrolyzing activity." Accordingly, in view of applicant's amendments to the claims, the claimed invention is enabled.

Accordingly, applicant respectfully requests that the rejection be reconsidered and withdrawn.

Claim 7 were rejected under 35 U.S.C. §112, first paragraph for alleged lack of written description. The examiner states that claim 7 recites the inclusion of a human β-1,3-glucanase in the human chitinase composition. However, the examiner contends that at the time of filing the instant application, there were no know human β-1,3-glucanase activity in any human cell line or tissue sample found in the prior art or in applicant's specification.

Applicant respectfully disagree. At page 24, lines 11-20 of the specification, applicant discloses that long term cultured macrophages secrete chitinolytic enzymes and an enzyme active against dye labeled β-glucan. The enzyme is referred to a β-glucanase, and its

mixture with human chitinase is proposed to be a more powerful anti-fungal agent than these enzymes alone.

Accordingly, in view of the above, applicant respectfully requests that the rejection of claim 7 under 35 U.S.C. §112 be withdrawn.

Claim 1 and 8 were rejected under 35 U.S.C. §102(b) as allegedly being anticipated by Overdijk et al. According to the examiner, Overdijk et al. teach the isolation of a human chitinase and compositions thereof.

Applicant respectfully disagree that the claimed invention is anticipated by Overdijk et al. First, nowhere is there any disclosure or suggestion of a human chitinase which comprises an amino acid sequence selected from the group consisting of an amino acid sequence depicted in SEQ ID NO:4, or an essentially corresponding form of said human chitinase having a sequence homology of at least 70%, having chitin-hydrolyzing activity.

In fact, Overdijk et al. disclose a human chitinase enzyme having an apparent molecular weight of 17 kDa as deduced from gel filtration (see page 797, col. 2, line 6 of Overdijk et al.). This enzyme is therefore very different from the chitinase disclosed in the present application. The chitinases of the present invention have molecular weights of about 50 kDa and 39 kDa.

As taught by Overdijk et al. (see page 802, col. 1, last 8 lines), such differences in apparent molecular weights demonstrate that the chitinases are unrelated. Therefore, according to the teachings of Overdijk et al., the chitinases of the present invention are unrelated to the human chitinase disclosed in Overdijk et al.

Accordingly, the claimed chitinase is patentable over Overdijk et al. Therefore, applicant respectfully requests that the rejection under 35 U.S.C. §102(b) be reconsidered and withdrawn.

Claims 1-4 and 9 were rejected under 35 U.S.C. §102(a) as allegedly being anticipated by Renkema et al. Further claim 30 were rejected under 35 U.S.C. §103(a) as allegedly being obvious over Renkema et al. The publication of Renkema et al. is a publication of the inventor, Aerts and is not available as prior art. See In re Katz, 687 F.2d 450, 215 USPDQ (CCPA 1982). The declaration of Johannes M.F.G. Aerts to establish this fact is appended hereto as exhibit 1.

Therefore, applicant respectfully requests that the rejections under both 35 U.S.C. §102(a) be reconsidered and withdrawn.

Claims 5, 6, and 9-12 were rejected under 35 U.S.C. §103(a) as allegedly being obvious over Renkema et al. or Overdijk et al. in view of Davies et al. or Pope et al. The examiner states that Renkema et al. and Overdijk et al. teach a human chitinase. Further, the examiner contends that Davies et al. and Pope et al. each teach that mycolytic enzymes, such as chitinases, are useful for the treatment of fungal infections. Therefore, the examiner alleges that it would have been obvious to make pharmaceutical compositions containing the chitinase of Renkema et al. and Overdijk et al. in products for inhibiting fungal activity.

Applicant has provided a declaration to remove the Renkema et al. reference as prior art (see above).

Further, applicant has pointed out above that the claimed chitinase is patentable over all the prior art, including Overdijk et al. Therefore, it would not be within the skill in the art to use the claimed chitinase in pharmaceutical compositions to treat fungal activity.

Accordingly, applicant respectfully requests that the rejection under 35 U.S.C. §103(a) be reconsidered and withdrawn.

Claims 12-14 were rejected under 35 U.S.C. §103(a) as allegedly being obvious over Renkema et al. or Overdijk et al. in view of Wheatley et al. (U.S. Patent 4,933,185). The examiner states that Renkema et al. and Overdijk et al. teach human chitinase. The examiner

further states that Wheatley et al. teach a controlled drug release system containing chitinase. Therefore, the examiner contends that it would have been obvious to make a controlled drug release system, as disclosed in Wheatley et al., comprising the chitinase of Renkema et al. or Overdijk et al.

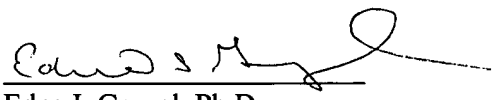
As stated above, applicant has provided a declaration to remove the Renkema et al. reference as prior art.

Further, applicant has pointed out above that the claimed chitinase is patentable over all the prior art, including Overdijk et al. Therefore, it would not be within the skill in the art to use the claimed chitinase in a controlled-drug release system, as disclosed in Wheatley.

Accordingly, applicant respectfully requests that the rejection under 35 U.S.C. §103(a) be reconsidered and withdrawn.

In view of the above amendments and remarks, allowance of the pending claims is earnestly requested. If the examiner has any questions regarding this amendment, the examiner is invited to contact the undersigned at the telephone number listed below.

Respectfully submitted,



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Human serum contains a chitinase: identification of an enzyme, formerly described as 4-methylumbelliferyl-tetra-*N*-acetylchitotetraoside hydrolase (MU-TACT hydrolase)

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Since 1988 an endoglucosaminidase, provisionally named MU-TACT hydrolase, has been known that hydrolyses the artificial substrate 4-methylumbelliferyl-tetra-*N*-acetylchitotetraoside (MU-[GlcNAc]$_4$, where GlcNAc is *N*-acetylglucosamine). The biological function of the enzyme was unknown. In this paper evidence is presented showing that this endoglucosaminidase from human serum is in fact a chitinase that is different from lysozyme. The facts sustaining this finding are: (i) the identification of the products formed from MU-[GlcNAc]$_3$ as [GlcNAc]$_2$ and [GlcNAc]$_3$; (ii) chitin and ethylene glycolchitin can be degraded by the enzyme; (iii) the chitinase inhibitor allosamidin also inhibits the action of MU-TACT hydrolase from human serum; (iv) no hydrolysis of the lysozyme substrate *Micrococcus lysodeikticus*. The enzyme also occurs in rat liver. It was demonstrated that upon Percoll density gradient centrifugation the enzyme from this tissue distributed parallel to the lysosomal marker enzymes β-*N*-acetylhexosaminidase and β-galactosidase, indicating a lysosomal localization for this enzyme. It is proposed that the enzyme functions in the hydrolysis of chitin, to which mammals are frequently exposed during infection by pathogens.

Key words: allosamidin/chitinase/human serum/lysozyme/MU-TACT hydrolase

Introduction

A novel enzyme that has activity towards 4-methylumbelliferyl-tetra-*N*-acetylchitotetraoside (MU-[GlcNAc]$_4$, where GlcNAc is *N*-acetylglucosamine) has recently been described (Den Tandt *et al.*, 1988, 1993; Overdijk *et al.*, 1994). Since we did not know the biological function of the enzyme, we provisionally named it MU-TACT hydrolase (4-methylumbelliferyl-tetra-*N*-acetylchitotetraoside hydrolase). It appeared to be present in human serum and plasma, but also in rat liver. The enzyme also shows activity with the di- and trimeric substrates MU-[GlcNAc]$_2$ and MU-[GlcNAc]$_3$. We ruled out the possibility that the MU-TACT hydrolase activity would in fact be exerted by a variety of other exo- or endoglycosidases, like β-*N*-acetylhexosaminidase (EC 3.2.1.30), acid chitobiase (formerly EC 3.2.1.29; now deleted), being a reducing-end exohexosaminidase (Baussant *et al.*, 1986; Kuranda and Aronson, 1986; Brassart *et al.*, 1987; Aronson *et al.*, 1989) and the endoenzymes hyaluronidase (EC 3.2.1.35), endoglucosaminidase D and H, the neutral endoglucosaminidase (EC 3.2.1.96) described in rat and human tissues (Pierce *et al.*, 1979, 1980; Overdijk *et al.*, 1981; Lisman *et al.*, 1985) and aspartylglucosaminidase (EC 3.5.1.26).

In order to find an answer to the question of the biological function of the enzyme, we purified it from human serum 56 000-fold (Overdijk *et al.*, 1994). The native enzyme appeared to have an apparent mol. wt of 17 kDa, as deduced from gel filtration.

With the partially purified enzyme preparation, we investigated the kinetics and biological function of MU-TACT hydrolase. It appeared that the enzyme is in fact a chitinase, having a lysosomal localization, as was shown upon subcellular fractionation studies of rat liver. So far only in goat and bovine serum had a chitinase of possible mammalian origin been described (Lundblad *et al.*, 1974, 1979). However, the molecular properties of the human enzyme are clearly different from those of the goat and bovine serum enzymes.

For reviews on chitin metabolism and chitinases, see Cabib (1987) and Flach *et al.* (1992).

Results and discussion

Enzyme kinetics with the substrate MU-[GlcNAc]$_3$

The purified human serum MU-TACT hydrolase was incubated for 15 and 60 min with increasing concentrations (0–100 μM) of MU-[GlcNAc]$_3$ substrate. The Michaelis–Menten curves that were constructed with the observed enzyme activities did not have the normal hyperbolic form, as is shown in Figure 1. Above a substrate concentration of 20–30 μM, inhibition was observed. The difference in the position of the observed maxima for the two incubation times can be explained by the degree of consumption of the substrate. In the upper curve (60 min), the observed activity for the lowest substrate concentration (16.7 μM) is clearly too low, since this value indicates that 95–100% of the initial amount of substrate has been consumed. A similar form of the Michaelis–Menten curve was observed when we repeated the above experiment with chitinase from *Streptomyces griseus* (data not shown). Gooday *et al.* (1988) also showed substrate inhibition for chitinase from females of the worm *Onchocerca gibsoni*. The latter authors used 3,4-dinitrophenyl-tetra-*N*-acetylchitotetraoside as a substrate. Substrate inhibition with glycol chitin as substrate was also seen for chitinase from the horn worm *Manduca sexta* (Koga *et al.*, 1983). In both the above references, the authors mention that conventional kinetic analysis was therefore inappropriate.

Reaction product analysis for the substrate MU-[GlcNAc]$_3$

From an earlier study, we knew that MU-TACT hydrolase can hydrolyse the MU derivatives MU-[GlcNAc]$_2$, MU-[GlcNAc]$_3$ and MU-[GlcNAc]$_4$. Furthermore, when using MU-[GlcNAc]$_4$ as substrate, the enzyme could be inhibited by the di-, tri- and

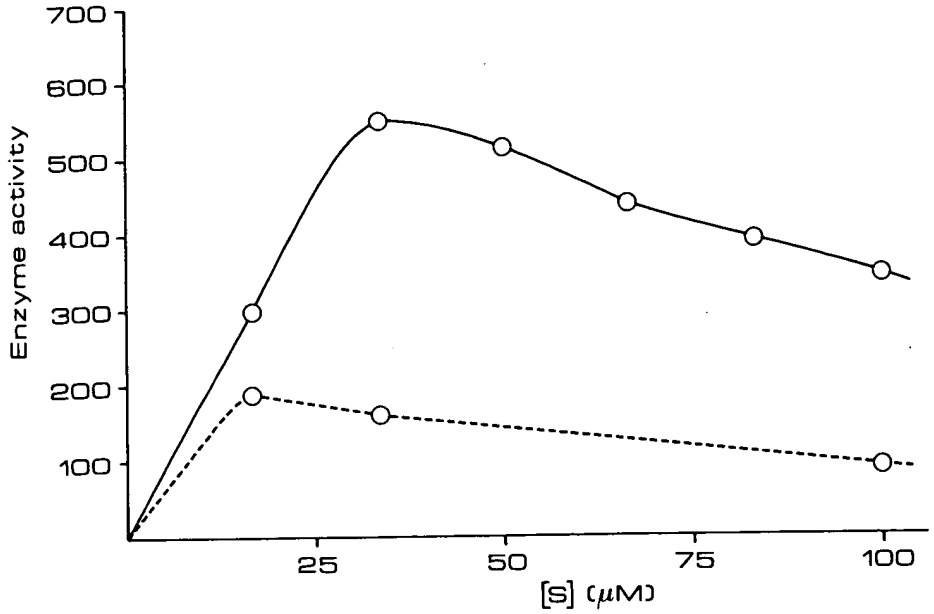

Fig. 1. Michaelis–Menten curves for MU-TACT hydrolase (chitinase) from human serum (20 μl; 16.4 μU), incubated with 30 μl buffered MU-[GlcNAc]$_3$ solution of varying concentrations for 60 min (—○ — ○—) and 15 min (-○----○-). The enzyme activities were measured as described in Materials and methods and are expressed in fluorescence values.

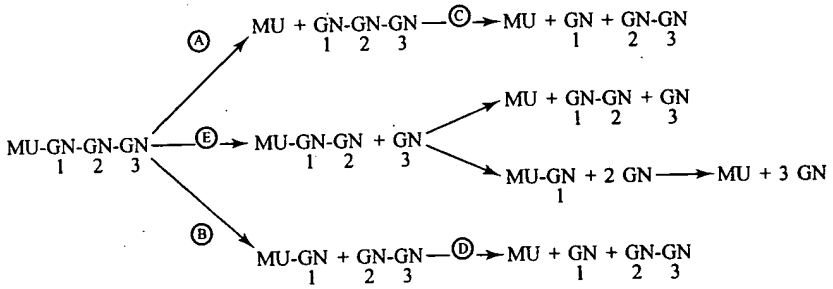

Fig. 2. Evaluation of the possibilities of enzymatic cleavage of MU-[GlcNAc]$_3$ by the action of MU-TACT hydrolase (chitinase). The enzyme can hydrolyse the substrate via steps A and B. The reaction products of step A will be further hydrolysed via step C, leading to a mixture of [GlcNAc]$_3$, [GlcNAc]$_2$ and GlcNAc. The reaction products of step B cannot be hydrolysed further by MU-TACT hydrolase, but the consecutive action of jack bean β-*N*-acetylhexosaminidase gives [GlcNAc]$_2$ and GlcNAc (GN = GlcNAc). For a discussion on the possibility of step E, see the text.

tetramers of GlcNAc (Den Tandt *et al.*, 1993). To find out whether MU-TACT hydrolase could hydrolyse more than one of the three glycosidic linkages present in MU-[GlcNAc]$_3$, we analysed the reaction products by HPLC, using a Lichrosorb-NH_2 column. It appeared that both [GlcNAc]$_2$ and [GlcNAc]$_3$ were formed from MU-[GlcNAc]$_3$. In Figure 2, we elaborate on the various possibilities of the mode of action of MU-TACT hydrolase that can yield the disaccharide. [GlcNAc]$_2$ could have been formed either directly with the concomitant formation of MU-[GlcNAc]$_1$ (step B) or after the prior formation of the intermediate products MU-[GlcNAc]$_2$ (step E), and/or [GlcNAc]$_3$ (step A). To find out which possibility was true, we added β-*N*-acetylhexosaminidase from jack bean to a reaction mixture resulting from the prior action of MU-TACT hydrolase. From earlier results, we knew that jack bean β-*N*-acetylhexosaminidase is virtually inactive against MU-[GlcNAc]$_2$ or MU-[GlcNAc]$_3$ (data not shown). Since MU was formed in the second incubation step with β-*N*-acetylhexosaminidase, the initial and simultaneous actions of MU-TACT hydrolase must have been (i) liberation of [GlcNAc]$_3$ (via step A) and (ii) formation of MU-GlcNAc and [GlcNAc]$_2$ (via step B). The other possibility in which MU-TACT hydrolase would have liberated GlcNAc by an exo-glycosidase action, thus yielding MU-[GlcNAc]$_2$ (step E), is highly improbable for the following reasons. From earlier work, we already knew that MU-TACT hydrolase is unable to hydrolyse MU-[GlcNAc]$_1$ (data not shown), and thus probably cannot split off the GlcNAc residue 3 of MU-[GlcNAc]$_3$. Furthermore, no endogenous, contaminating β-*N*-acetylhexosaminidase activity could be measured in the MU-TACT hydrolase preparation.

Degradation of chitin and ethylene glycol [^{3}H]chitin

When we incubated a suspension of chitin with human serum MU-TACT hydrolase or with chitinase from *S.griseus*, hydrolysis of this substrate was observed, as is shown in Figure 3. The same reaction products were obtained with both enzymes, proving the chitinase character of the human serum enzyme. However, the ratios of the amounts of the reaction products [GlcNAc]$_1$, [GlcNAc]$_2$ and [GlcNAc]$_3$ differed.

With tritiated ethylene glycolchitin, prepared by [^{3}H]-acetylation of ethylene glycolchitosan, we also clearly observed

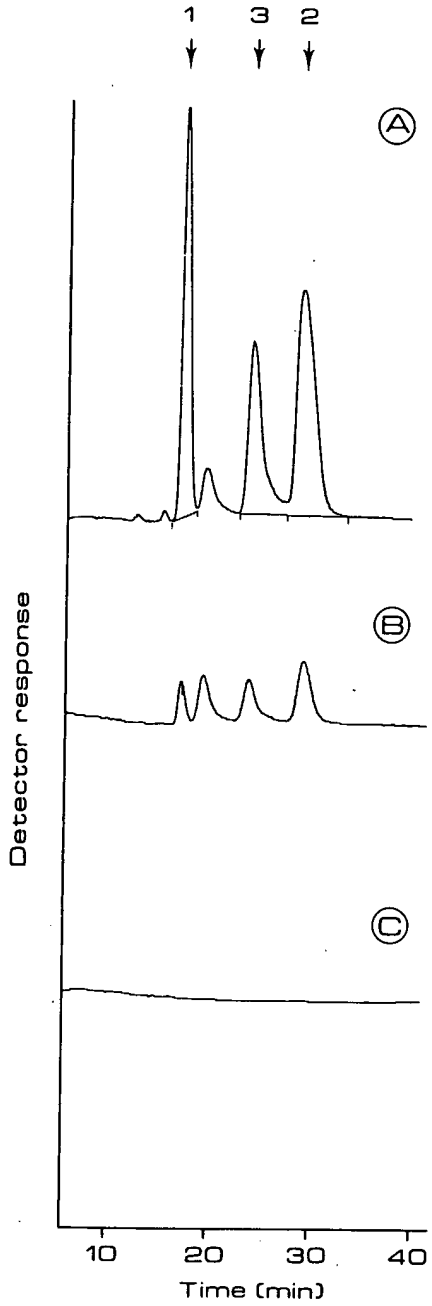

Fig. 3. HPAEC-PAD of the reaction products of the hydrolysis of chitin by chitinase from *S.griseus* (**A**) and by human serum MU-TACT hydrolase (chitinase) (**B**). The numbered peaks are $[GlcNAc]_1$ (1), $[GlcNAc]_2$ (2) and $[GlcNAc]_3$ (3). Identification of these peaks was done by means of reference substances. The identity of the peak between (1) and (3) is not known. It could be $[GlcNAc]_4$, but we had no reference available for that substance. For experimental details regarding the hydrolysis, see Materials and methods. (**C**) represents an experiment without addition of enzyme to the substrate.

hydrolysis of this material by MU-TACT hydrolase and *S.griseus* chitinase (data not shown).

Affinity chromatographic behaviour of MU-TACT hydrolase on a chitin column

In a paper by Jensen and Kleppe (1972), lysozyme from T4-infected *Escherichia coli* cells was purified on a column of

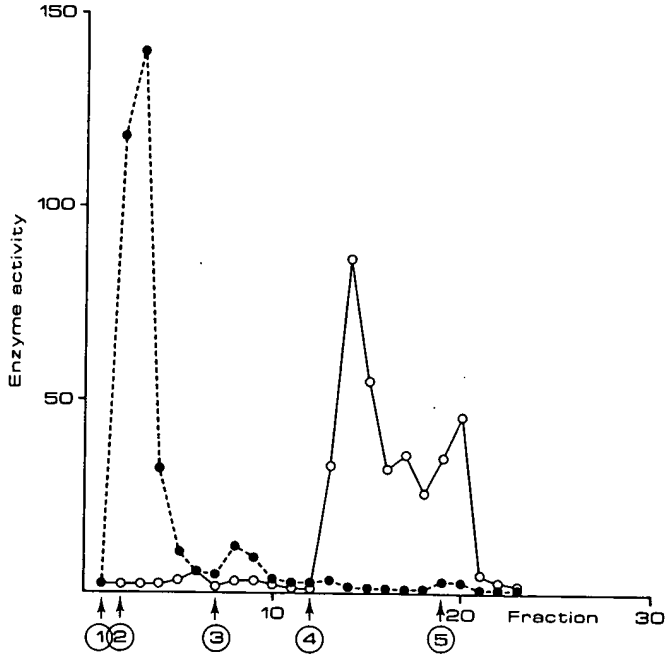

Fig. 4. Chitin affinity chromatography of human serum MU-TACT hydrolase (chitinase). A column (38 × 5 mm) was filled with pre-treated chitin, suspended in 0.05 M Na-phosphate buffer (pH 7.0). A sample of human serum MU-TACT hydrolase (1 ml; 0.68 mU) was applied (arrow 1). The column was run at a flow rate of 1.75 ml/h in three steps: (i) 0.05 M Na-phosphate buffer, pH 7.0 (4.35 ml; arrow 2); (ii) 0.05 M sodium acetate buffer, containing 0.25 M KCl and 0.1% (w/v) Triton X-100, pH 4.6 (4.35 ml; arrow 3); (iii) 0.1% (w/v) Triton X-100 in water (10.45 ml; arrow 4). Elution started at arrow 4 and was interrupted overnight and continued at arrow 5. Enzyme activity is expressed in arbitrary units; recovery of the activity was 109%. MU-TACT hydrolase (—○—○—); protein (-●----●-).

chitin. Such columns have also been successfully used for the purification of chitinases [refs 16, 73, 112, 133 and 155 in Flach *et al.* (1992)]. For the characterization of our enzyme, we found it worthwhile to see if it would also be bound by such an affinity column. For that purpose we pre-treated chitin (practical grade) as described by Jensen and Kleppe (1972) and filled a small column with it. The column was eluted essentially as described by the latter authors (see Materials and methods). It appeared that MU-TACT hydrolase was retained by the column and could be eluted with water (Figure 4), similarly to what was found for hen egg-white lysozyme (Cherkasov and Kravchenko, 1970; see also ref. 11 therein).

Activity of MU-TACT hydrolase and chitinase with the lysozyme substrate Micrococcus lysodeikticus

Since MU-TACT hydrolase, chitinase and lysozyme all bind to a chitin column, it was of interest to find out whether MU-TACT hydrolase and chitinase would show activity with the lysozyme substrate *Micrococcus lysodeikticus*. For that purpose we incubated 0.5 mg of the bacterial cells with 2.5 μg of the following enzymes: human serum MU-TACT hydrolase, chitinase from *S.griseus* and lysozyme from hen egg white, under conditions that were essentially identical with those described by Jollès (1962) for the assay of lysozyme. While lysozyme showed an increase in transmission (ΔT) at 466 nm of 9%/min, there was virtually no effect with MU-TACT hydrolase and chitinase. Even prolonged incubation for 1 h resulted

Table I. Inhibition (%) by ethylene glycolchitin and ethylene glycolchitosan of the hydrolysis of MU-[GlcNAc]$_3$ by human serum MU-TACT hydrolase and chitinase of *S.griseus*

Enzyme	Ethylene glycolchitin	Ethylene glycolchitosan
MU-TACT hydrolase (22 μU)	98	43
Chitinase (10 μU)	96	63

The concentration of both above 'inhibitors' was 0.4 mg/ml. The differences in the percentages for the chitin and chitosan derivative can easily be explained by the difference in the molecular nature and by the lower degree of polymerization of the chitin derivative.

in ΔT values of 0%, although with such long incubation periods there was no longer linearity with lysozyme. During that time period, the latter enzyme showed a ΔT of 59%. The above result confirms the earlier observation that human serum MU-TACT hydrolase and lysozyme are different enzymes (Den Tandt *et al.*, 1988; see also the next paragraph).

Inhibition of MU-[GlcNAc]$_3$ hydrolysis

When we added the water-soluble substances ethylene glycolchitosan or ethylene glycolchitin to a standard reaction mixture of the human serum enzyme with MU-[GlcNAc]$_3$ as a substrate, we clearly observed a significant inhibition of the liberation of 4-methylumbelliferone (MU). The same effect was observed for chitinase from *S.griseus* (Table I).

Koga *et al.* (1987) have shown that allosamidin, a dimeric derivative of the GlcNAc analogue *N*-acetylallosamine, is a competitive inhibitor of chitinase. The concentrations that are inhibitory differ very much for the various chitinases reported, from the picomolar to the micromolar range (Gooday *et al.*, 1988). When we used allosamidin to see whether it would have an effect on the activity of MU-TACT hydrolase, we found a 50% inhibition of the enzyme activity at ~5.6 nM under standard assay conditions for the enzyme (Figure 5). A similar experiment with chitinase from *S.griseus* showed 50% inhibition at an approximate concentration of 15 nM (Figure 5).

Since the MU-[GlcNAc]$_3$ substrate can be hydrolysed by lysozyme if high concentrations of this enzyme are present, it was of interest to see whether lysozyme could also be inhibited by the chitinase inhibitor allosamidin. It appeared that this compound, in a concentration range of 0–5 × 10^5 pM, was not inhibitory for lysozyme from chicken egg white or from human serum, which is in agreement with earlier results by Koga *et al.* (1987) (see Figure 5). This again confirms the non-identity of MU-TACT hydrolase and lysozyme.

Subcellular localization of rat liver MU-TACT hydrolase

The results of the subcellular fractionation of a rat liver homogenate are shown in Table II. The soft upper part of the P_2 pellet ($P_{2,S}$) was used in the subsequent Percoll gradient centrifugation step. The data of Table II indicate that the lysosomal marker enzymes β-galactosidase and β-*N*-acetylhexosaminidase are enriched when compared with the homogenate by a factor of 1.8–1.9. For MU-TACT hydrolase,

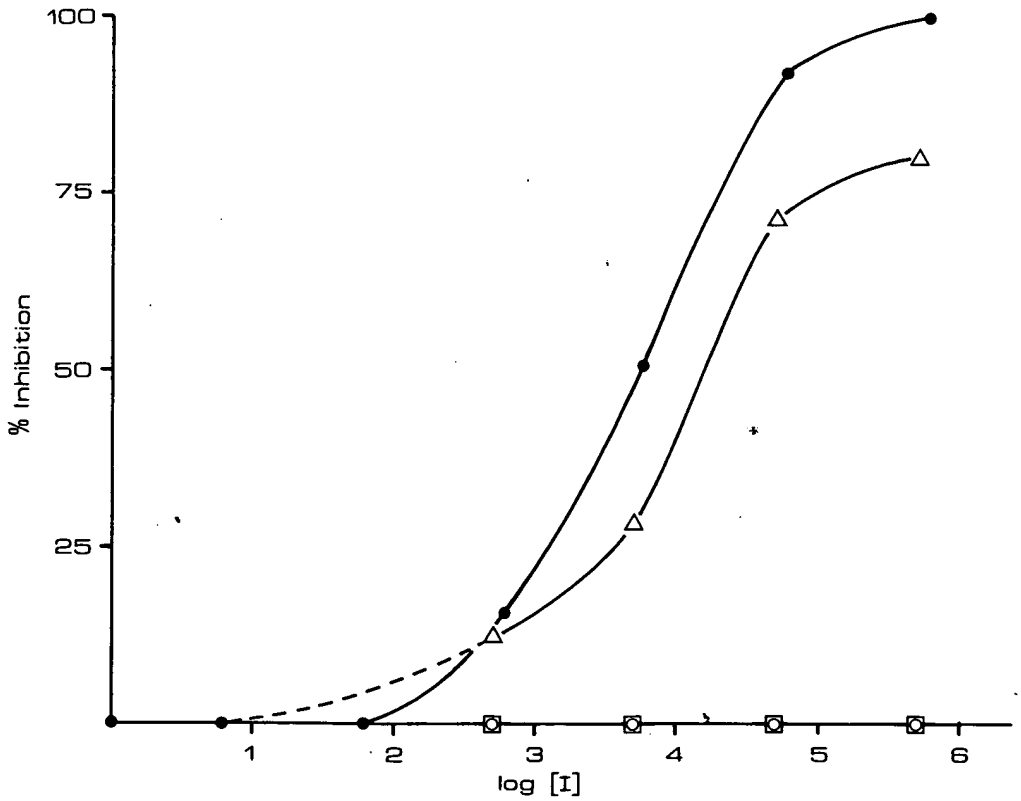

Fig. 5. Inhibition by allosamidin of human serum MU-TACT hydrolase (4.5 μU), chitinase from *S.griseus* (10 μU), hen egg white lysozyme (8160 U) and human serum lysozyme (37 000 U). With these amounts of enzyme, comparable activities towards the MU-[GlcNAc]$_3$ substrate have been used. For definitions of the enzyme activity units, see Materials and methods. Inhibitor concentrations (pM) have been plotted on a logarithmic scale on the abscissa. MU-TACT hydrolase (—●—●—); chitinase (—△—△—); hen egg white lysozyme (—□—□—); human serum lysozyme (—○—○—). The dashed part of the chitinase curve forms an extrapolation to a zero concentration (log [*I*] undefined) of allosamidin.

Table II. Subcellular fractionation of rat liver MU-TACT hydrolase (chitinase)

Marker	Protein		β-Hex			β-Gal			SDH			MU-TACT		
Fraction	Level	%	SA	%	RSA	SA	%	RSA	SA	%	RSA	SA	%	RSA
Homogenate	17.4 ± 4.7	100	25.8 ± 0.3	100	1.00	2.9 ± 0.0	100	1.00	21.1 ± 0.1	100	1.00	1.3 ± 0.0	100	1.00
S_1	79.9 ± 1.2	46.6	32.6 ± 1.4	59.0	1.26	3.7 ± 0.1	59.1	1.28	2.5 ± 0.2	5.4	0.12	1.6 ± 0.1	55.2	1.23
P_1	71.4 ± 1.7	41.7	16.7 ± 0.4	26.9	0.65	1.9 ± 0.0	27.3	0.66	25.8 ± 0.6	51.00	1.22	1.1 ± 0.1	33.3	0.85
S_2	40.8 ± 2.9	23.8	1.0 ± 0.1	0.9	0.04	0.6 ± 0.1	4.8	0.21	0.4 ± 0.0	0.5	0.02	0.9 ± 0.0	15.2	0.69
P_{2D}	<1.0	–	nd	15.3	–	nd	13.6	–	nd	0.4	–	nd	15.6	–
P_{2S}	36.7 ± 1.1	21.4	48.7 ± 0.7	40.5	1.89	5.1 ± 0.1	37.6	1.76	7.7 ± 0.1	7.9	0.36	1.5 ± 0.0	23.6	1.15

Units used in the table are: protein level: mg/g of tissue; specific activities (SA): mU/mg protein [for β-*N*-acetylhexosaminidase (β-Hex) and β-galactosidase (β Gal), U/mg protein (for succinate dehydrogenase (SDH)], μU/mg protein (for MU-TACT hydrolase). RSA = relative specific activity. The fractions were prepared according to Caimi *et al.* (1989). Briefly, the mentioned fractions are: S_1, P_1 (supernatant and pellet of 10 min, 1000 *g* centrifugation of the homogenate); S_2, P_2 (supernatant and pellet of 60 min, 17 500 *g* centrifugation of the S_1 fraction; the indices 'S' and 'D' of P_2 denote the upper soft part and the lower dense part of the pellet, respectively).

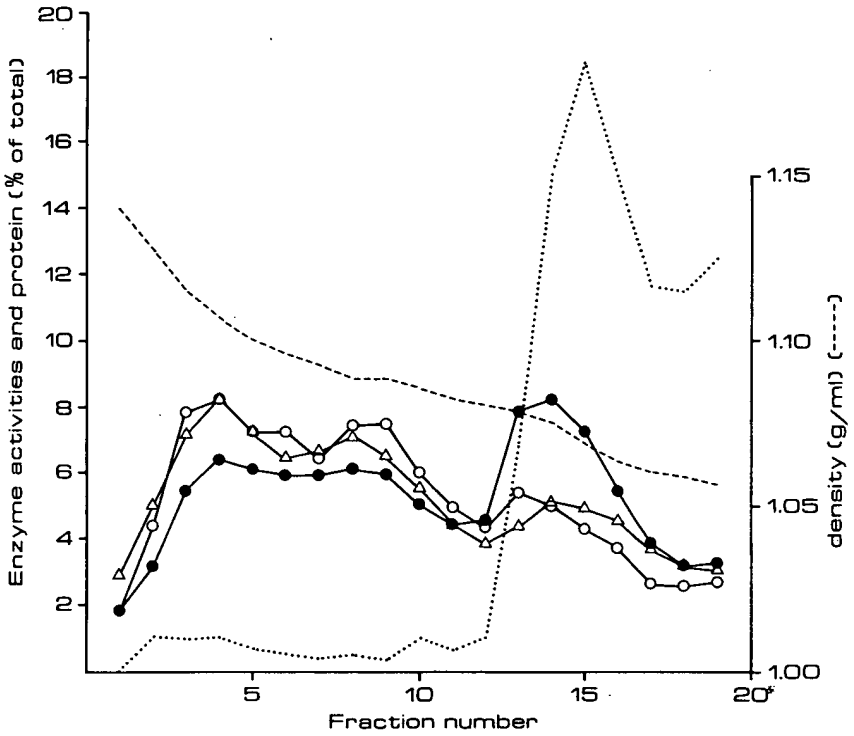

Fig. 6. Isopycnic centrifugation on a 37.4% self-generating Percoll gradient of a crude lysosomal–mitochondrial pellet from a rat liver homogenate. Distribution of MU-TACT hydrolase and some marker enzymes. For experimental details, see Materials and methods. MU-TACT hydrolase (—●—●—); β-*N*-acetylhexosaminidase (—△—△—); β-galactosidase (—○—○—); protein (····); density, determined by refractive index determination (— — —).

the enrichment was lower (1.15). This difference in enrichment factor is probably caused by the fact that a relatively high percentage of the MU-TACT hydrolase activity was present in the S_2 fraction (15.2 versus 0.9% for β-*N*-acetylhexosaminidase and 4.8% for β-galactosidase). When the $P_{2,S}$ fraction was subsequently fractionated on a Percoll gradient with a starting density of 1.086 g/ml, we obtained the following results (Figure 6). The lysosomal marker enzymes β-*N*-acetylhexosaminidase and β-galactosidase were distributed over the gradient in two zones, representing the well-known light (density 1.07–1.08) and heavy (density 1.09–1.11) lysosomes that have been described for various cell types (Rome *et al.*, 1979; Miller *et al.*, 1986, 1993; Little *et al.*, 1987; Chang *et al.*, 1988; Caimi *et al.*, 1989; Kelly *et al.*, 1989; see also Table III). The zone of the highest density was comprised of two peaks with their tops in fractions 4 and 8, respectively. When a starting density of 1.075 g/ml was used, we found only one peak in that density region at 1.10–1.11 g/ml (data not shown). As is clear from Figure 6, MU-TACT hydrolase co-sediments with the lysosomal markers β-galactosidase and β-*N*-acetylhexosaminidase, ending up in both lysosomal populations. Compared with the latter marker enzymes, a higher

Table III. Densities of heavy and light lysosomes from various sources, separated by Percoll self-generating density gradient centrifugation

Cell/tissue	Authors	Density (g/ml)		Starting density of Percoll in 0.25 M sucrose
		Light lysosomes	Heavy lysosomes	
Human fibroblasts	Kelly *et al.*, 1989	1.10	1.11	27%
	Rome *et al.*, 1979	1.085	1.11	–
Human lymphoblasts	Miller *et al.*, 1993	1.088	1.12	1.065 g/ml
Mouse brain	Caimi *et al.*, 1989	1.07	1.11–1.12	1.07 g/ml
Rat liver	Pertoft and Wärmgård, 1978	1.06–1.07	1.09–1.10	1.077 g/ml
	Present work (data not shown)	1.07	1.10–1.11	1.075 g/ml
	Present work (data taken from Figure 6)	1.07–1.08	1.09;1.10–1.11	1.086 g/ml

proportion of MU-TACT hydrolase is present in the light lysosomal fraction. The percentage distribution given in Table II shows that for MU-TACT hydrolase a higher part of the enzyme activity present in the P_2 fraction sedimented in the $P_{2,D}$ part of it. This part of the pellet will contain a relatively high amount of heavy lysosomes. Therefore the difference in distribution of MU-TACT hydrolase over light and heavy lysosomes could have been different if the pellets $P_{2,S}$ and $P_{2,D}$ had been taken together. The above results clearly show that in rat liver MU-TACT hydrolase is a lysosomal enzyme.

In summary, we have shown that the recently described novel endoglucosaminidase in human serum, that was given the provisional name MU-TACT hydrolase, is in fact a chitinase and is definitively different from lysozyme. This is the first time that a human chitinase has been described.

One could speculate on the biological function of mammalian chitinases. Flach *et al.* (1992) summarized the functions of chitinases in various organisms, like plants, fungi, bacteria, insects, marine invertebrates and fishes. In some sources, the enzyme is present constitutively, whereas in others, like plants and bacteria, the activity arises after induction. It is proposed that the enzyme functions in the hydrolysis of chitin, to which an organism is frequently exposed during infection by pathogens. It could, therefore, be possible that in mammals the presence of chitinase also forms such a defence mechanism against pathogens.

The human enzyme that is described here and in earlier papers (Den Tandt *et al.*, 1988, 1993; Overdijk *et al.*, 1994) is probably unrelated to the formerly described bovine or goat serum enzymes, since those enzymes have a much higher apparent mol. wt in gel filtration of 47 and 60 kDa, respectively (Lundblad *et al.*, 1974, 1979). Furthermore, the bovine enzyme also exhibited kinetics and a behaviour on an anion exchanger that were different from that of the human enzyme (Overdijk and Van Steijn, manuscript in preparation). Work is in progress to find out the function of chitinase in human tissues and blood.

Materials and methods

Materials

Superose 12 HR and Percoll (1.130 g/ml) were from Pharmacia LKB Biotechnology, Uppsala, Sweden. Chitinase from *S.griseus* (0.5 Sigma U/mg solid), hen egg white lysozyme (40 800 Sigma U/mg solid), β-*N*-acetylhexosaminidase from jack bean, chitin from crab shells (both the grade suitable for the assay of chitinase and also the practical grade crude powder for chromatographic purposes), ethylene glycol chitosan (degree of polymerization 810), *M.lysodeikticus* (lysozyme substrate), 4-methylumbelliferyl-tri-*N*-acetylchitotrioside (MU-[GlcNAc]$_3$) and 4-methylumbelliferyl-*N*-acetyl-D-glucosaminide (MU-[GlcNAc]$_1$) were from Sigma Chemical Co., St Louis, MO. Human serum lysozyme (185 000 U/mg, as determined with the Behring Testomar-lysozyme kit), was a kind gift of Dr J.Brouwer, Delft, The Netherlands. Ethylene glycol chitin was from Seikagaka Corp., Tokyo, Japan. Allosamidin from *Streptomyces* sp. 1713 was a kind gift of Drs A.Suzuki and A.Isogai, Department of Agricultural Chemistry, University of Tokyo, Japan. The substance is composed of two β-linked *N*-acetylallosamine residues that are β-glycosidically linked to allosamizoline (Sakuda *et al.*, 1986, 1988). *N*-acetylallosamine differs from GlcNAc in the configuration of the hydroxyl group at the C_3-position. Allosamizoline is an aminocyclitol derivative. Triton X-100 was from Merck, Darmstadt, FRG. MU-TACT hydrolase, purified from human serum as described elsewhere (Overdijk *et al.*, 1994), had a specific activity of 250 U/g. BCA (bicinchoninic acid) protein assay reagent was from Pierce Europe, Oud-Beijerland, The Netherlands. All other chemicals were of the best available grade.

Chitin degradation and product analysis

With chitin as substrate, the enzymatic activities of the commercial chitinase from *S.griseus* and of human serum MU-TACT hydrolase were determined as follows. Chitin (3.3 mg; analytical grade) was suspended in 300 µl 1.0 M sodium acetate buffer (pH 5.5) containing 0.02% NaN_3, by ultrasonification. Chitinase (250 mU; 0.5 mg) was dissolved in 200 µl 0.02 M sodium acetate buffer, containing 0.1 M NaCl, 0.02% NaN_3 and 0.1% (w/v) Triton X-100 (pH 5.5). The chitin suspension (30 µl) was mixed with 20 µl of either the above chitinase solution or of a similar solution of human serum MU-TACT hydrolase (2.6 mU). The mixtures were incubated at 37°C for 21 h and then centrifuged for 5 min at 12 000 *g*. To the supernatants (40 µl) were added 160 µl water and 15 µl human serum albumin (10 mg/ml), followed by 80 µl trichloroacetic acid (TCA) (10% w/v). The mixtures were kept on ice for 15 min and were then centrifuged for 10 min at 12 000 *g*. The supernatants (250 µl) were neutralized by adding 10 µl 2.0 M NaOH. The reaction products, present in these supernatants, were identified by high-performance anion-exchange chromatography with a CarboPac PA-1 column (HPAEC-PAD), as described by Nemansky *et al.* (1992).

Analysis of the products of hydrolysis of MU-[GlcNAc]$_3$

Identification of the products of hydrolysis of the substrate MU-[GlcNAc]$_3$ was performed by HPLC on a Lichrosorb-NH_2 column, as described by Blanken *et al.* (1985).

Chitin affinity chromatography

Chitin (practical grade) was pre-treated essentially as described by Jensen and Kleppe (1972). Briefly, the chitin was powdered in a mortar and sieved through a nylon filter. The non-retained material was washed at 50°C on a glass filter with 1% solutions of NaCl and acetic acid, followed by washes with 10 mM EDTA (at 23°C), 0.05 M sodium acetate buffer, containing 0.25 M KCl, pH 4.6 (at 23°C) and 0.05 M sodium phosphate buffer, pH 7.0 (at 5°C), respectively. A column (38 × 5 mm) was filled with a suspension of the resulting retentate. A sample of human serum MU-TACT hydrolase in the latter buffer (1 ml; 0.68 mU) was applied to the column. The column was washed with 0.05 M sodium phosphate buffer, pH 7.0 (4.35 ml), followed by 0.05 M sodium acetate buffer, containing 0.25 M KCl and 0.1% (w/v) Triton X-100, pH 4.6 (4.35 ml). Elution was achieved with 10.45 ml water, containing 0.1% (w/v) Triton X-100. The flow rate was 1.75 ml/h.

Subcellular fractionation

The preparation of the lysosomal fraction of rat liver by sedimentation centrifugation was carried out essentially as described for mouse brain by Caimi *et al.* (1989). In our case, the lysosomal pellet P_2 was composed of two layers: a very small dense one ($P_{2,D}$) on the bottom of the centrifuge tube and a larger soft part ($P_{2,S}$) on top of the dense one. The latter material, suspended in 0.25 M sucrose containing 1 mM EDTA, was subsequently subfractionated on a self-generating Percoll gradient in 0.25 M sucrose (9 ml; 37.4%; starting density 1.085 g/ml) in a 10 ml centrifuge tube. A sample of 1 ml was layered on top of the Percoll suspension. After centrifugation for 90 min at 20 000 *g* in a fixed-angle rotor, 19 fractions were collected from the bottom of the tube. In each fraction, protein and the activity of MU-TACT hydrolase were determined, together with the activities of the following marker enzymes: β-galactosidase, β-*N*-acetylhexosaminidase, succinate dehydrogenase [for assay methods of the latter enzymes, see Lisman *et al.* (1978)].

Other assays

MU-TACT hydrolase activity was determined as described previously (Den Tandt *et al.*, 1988). One unit will liberate 1 μmol MU/min from MU-$[GlcNAc]_3$. Lysozyme activity with *M.lysodeikticus* as substrate was determined according to Jollès (1962). Protein was determined with the BCA method according to the manufacturer's instructions in the cases where Triton X-100 was present. In other cases, the Lowry method was used (Lowry *et al.*, 1951).

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Abbreviations

BCA, bicinchoninic acid; β-Gal, β-galactosidase; GlcNAc, *N*-acetylglucosamine; β-Hex, β-*N*-acetylhexosaminidase; HPAEC-PAD, high-performance anion-exchange chromatography with a CarboPac PA-1 column; MU, 4-methylumbelliferone; MU-$[GlcNAc]_n$, 4-methylumbelliferone linked to the reducing end of the (β1-4)-oligomer of *N*-acetylglucosamine; MU-TACT hydrolase, 4-methylumbelliferyl-tetra-*N*-acetylchitotetraoside hydrolase; SDH, succinate dehydrogenase; TCA, trichloroacetic acid.

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The influence of carbohydrases on the growth of fungal pathogens *in vitro* and *in vivo*

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Summary

Mixtures of mycolytic enzymes from various sources release protoplasts from living fungal tissue under suitable conditions. Such enzyme mixtures obtained from *Coprinus comatus* (mycolase I), *Physarum polycephalum* (mycolase II) and *Lycoperdon pyriforme* (mycolase III) are of low toxicity in mammals when given parenterally and are able to cure experimental systemic fungal infections in mice when administered alone or in conjunction with normally ineffective levels of conventional antimycotic drugs such as amphotericin B. The effect is believed to be due to enzymic degradation of the fungal cell wall either killing the fungus directly or enhancing activity of existing antifungal agents by increasing access to the cell interior.

Introduction

Fungal infections are a major cause of morbidity and mortality throughout the world (Ajello, 1971; Mycoses, 1975) and patients under induced immunosuppression for organ transplantation or receiving chemotherapy for cancer are susceptible to disease caused by 'opportunistic' organisms such as *Aspergillus* and *Candida* spp. (Bodey, 1977; Mason *et al.*, 1976). In spite of increasing awareness of the importance of fungal infections, there is a shortage of effective antimycotic agents and those that are available have drawbacks that limit their usefulness. Amphotericin B has been the drug of choice for treatment of systemic fungal disease, but it is nephrotoxic and has other undesirable side effects (Bennett, 1974). Flucytosine is less toxic than amphotericin but emergent fungal resistance to this drug is a potentially serious problem (Utz, 1977). Newer antifungal agents such as miconazole and clotrimazole are still being evaluated.

The essence of effective chemotherapy is to exploit differences between host and pathogen so that the host is unaffected by the treatment. In fungal infections, exploitable differences are few because both host and pathogen are eukaryotes. However, an obvious difference between fungal and mammalian cells that has not been exploited is the presence in fungi of a polysaccharide cell wall that frequently contains chitin (Bartnicki-Garcia, 1968). The cell wall can also be seen as a barrier to effective chemotherapy. Certainly, Gale *et al.* (1975) have shown that protoplasts of *C. albicans* are more sensitive to amphotericin B than are intact cells. The novel approach to antimycotic therapy described in this communication exploits the cell wall by making chitin and other polysaccharides the target for enzymatic attack. Damage to or removal of the cell wall should either kill the fungus directly or, at least, enhance the activity of existing drugs by increasing their access to the cell interior. Experiments intended to test this hypothesis are described below.

Materials and methods

Organisms and media

A. fumigatus and *C. albicans* were obtained from the Searle culture collection. *Physarum polycephalum* (strain i x a7029) was obtained from Dr M. J. Carlile, Department of Biochemistry, Imperial College of Science and Technology, Prince Consort Road, London, S.W.7. Specimens of *Coprinus comatus* and *Lycoperdon pyriforme* were collected in the Chiltern beechwoods around High Wycombe and stored at −20°C until required.

A. fumigatus and *Candida albicans* were grown on glucose peptone medium containing (per litre) glucose 10 g, peptone 2 g, KH_2PO_4 0·5 g, $MgSO_4$ 0·5 g. Universal bottles containing 10 ml of this medium were inoculated with a spore suspension of *A. fumigatus* and incubated for 20 hr at 24°C on a Luckham Rolamix blender. *P. polycephalum* was grown on the medium described by Carlile (1971).

Enzyme preparation

Enzyme mixtures were obtained from fresh or frozen specimens of *Coprinus comatus* and *L. pyriforme* either by allowing the fruit bodies to autolyse at 4°C in sterile containers for 24–48 h or by homogenizing fruit bodies in a Waring Blender for 5 min at 4°C. The homogenates obtained were expressed through muslin and the remaining solid discarded. The fluid was then centrifuged in sterile containers at 3000 *g* for 15 min and the pellet discarded. The supernatant contained the crude enzyme mixture which was then lyophilized.

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Protoplast assay

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Experimental infection

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TABLE 1. Production of pr
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are given in Table 1. Chiti

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A 'purified' extract of *L. pyriforme* was prepared by fractionating the crude enzyme mixture on Bio-Gel P200 (Bio-Rad Laboratories, Richmond, California) and recombining the retarded fractions.

A mixture of mycolytic enzymes was obtained from culture supernatants of *P. polycephalum* as follows: Cellular material was removed by centrifugation at 1600 *g* for 15 min at 4°C and viscous polysaccharide precipitated from the supernatant by adding one volume of cold (−20°C) 95% ethanol and centrifuging at 10 000 *g* for 30 min at 4°C to sediment the resultant precipitate. The clear supernatant was dialysed against water to remove the ethanol and lyophilized.

Chitinase (E.C. 3.2.11.4.) was obtained from Koch Light Laboratories, Colnbrook, Buckinghamshire, and laminarinase (β-1,3(4) glucan glucanohydrolase E.C. 3.2.1.6.) was obtained from Calbiochem, San Diego, California.

Protoplast assay

This was carried out according to the method of Bartnicki-Garcia and Lippmab (1966) using molar $MgSO_4$ as an osmotic stabilizer. *A. fumigatus* was the target organism and all enzymes were at a final concentration of 240 mg/l.

Experimental infection

Groups of 5 or 10 female BALB/c mice weighing 22–25 g were infected by intravenous injection with either 5×10^6 spores of *A. fumigatus* or 25×10^4 blastospores of *C. albicans* in saline. On one or more of the days following infection, animals were treated intraperitoneally with enzymes, conventional antimycotic drugs or combinations of the two. Treatments were given in 0·2 ml of 5% dextrose or water for injection.

TABLE 1. Production of protoplasts from *Aspergillus fumigatus*

Enzyme treatment	Protoplast release
Chitinase	+
β-1,3-glucanase	–
Chitinase + β-1,3-glucanase	+ +
Coprinus extract – mycolase I	+ + +
Physarum culture SN – mycolase II	+ + + + +
Lycoperdon extract – mycolase III	+ + +

\+ Protoplasts from hyphal tips only.
\+ + Protoplasts from tips and intercalary compartments.
\+ + + Many protoplasts released throughout mycelium.
\+ + + + + Total lysis of mycelium.

Results

Results for protoplast release from *A. fumigatus* are given in Table 1. Chitinase released protoplasts only from hyphal tips, whereas β-1, 3-glucanase did not liberate any protoplasts. A mixture (1 : 1 by weight) of the 2 enzymes was more effective than either component alone. Mycolases I, II and III (from *Coprinus comatus, P. polycephalum* and *L. pyriforme* respectively) were more effective than the commercial enzyme mixture, and treatment with mycolase II resulted in complete lysis of the target mycelium. Similar results have been obtained with *C. albicans* and a range of microfungi (Pope and Davies, unpublished data).

Results in Table 2 show that infected, untreated mice had a mean survival time of 13·5 days (Table 2 a). Under the conditions of the test, a single treatment with the maximal sub-toxic dose of amphotericin B or nystatin significantly prolonged survival, but did not cure the animals (Table 2 k, m). Animals were cured by mycolases alone (Table 2 d, j) but not by the commercial enzyme mixture (Table 2 b). Infections were also cured by the synergistic effect of mycolases and conventional antifungal agents at doses of both components that were ineffective when administered alone (Table 2 o, p, q, r, s). Mice that had been immunosuppressed with rabbit anti-mouse thymocyte globulin (ATG) were also cured by combined therapy, suggesting that a full immune capacity is not an essential prerequisite for the effect. Similar results have been obtained in experimental candidiasis and in the treatment of dermatophyte infection in guinea-pigs (Pope and Davies, unpublished data).

Acute toxicity tests with mycolases given intravenously or intraperitoneally in mice gave an LD_{50} of 15–20 mg/mouse (600–700 mg/kg).

Discussion

The fungal cell wall has been recognized by other workers as a potential target for therapeutic attack. Specific inhibitors of chitin synthetase, the polyoxins, have been developed (Endo, Kakiki and Misato, 1970), but although these compounds are effective *in vitro*, they have not proved useful clinically. Chitinases occur widely in nature (Stirling, Cook and Pope, 1979) and, although their effects on fungal cell walls *in vitro*, alone and in conjunction with other enzymes are well known, as far as the authors are aware there have been no *in vivo* studies. Miura (1954) suggested that chitinase might be used for treating dermatophyte infections, but did not demonstrate mycocidal activity. The results show that chitinase alone is only slightly mycolytic and requires the presence of other enzymes before its full potential can be realized. The increased mycolytic activity of mycolases compared with the commercial enzyme mixture is almost certainly attributable to the wider range of carbohydrases they contain. Enzyme profiles for mycolases will be published elsewhere.

TABLE 2. Fate of BALB/c mice infected systemically with *Aspergillus fumigatus*

Treatment	Days after challenge	Dose	Mean survival (days)
a. None (controls)	—	—	13·5
b. Chitinase + β-glucanase	1, 2, 3	3 × 1·5 mg	26·3
c. Mycolase I (crude)	1	1·5 mg	21·0
d. ,, ,,	1, 2, 3	3 × 1·5 mg	All survived*
e. ,, ,,	12	4·5 mg	35·6
f. Mycolase II (crude)	1	100 μg	16·2
g. ,, ,,	1	10 μg	14·4
h. Mycolase III (crude)	1	1·5 mg	11·2
i. ,, (purified)	1	100 μg	15·8
j. ,, ,,	1, 2, 3	3 × 100 μg	All survived*
k. Amphotericin B	1	5·0 μg	23·2
l. ,, ,,	1	0·6 μg	16·0
m. Nystatin	1	100 μg	30·6
n. ,,	1	12·5 μg	14·6
o. Mycolase I – amphotericin (c–l)	1–2	1·5 mg–0·6 μg	All survived*
p. Mycolase I – nystatin (c–n)	1–2	1·5 mg–12·5 μg	All survived*
q. Mycolase II – amphotericin (f–l)	1–2	100 μg–0·6 μg	All survived†
r. Mycolase II – amphotericin (g–l)	1–2	10 μg–0·6 μg	42·0
s. Mycolase III – amphotericin (h–l)	1	1·5 mg + 0·6 μg	All survived†
t. ATG‡ before challenge	−1	300 μl	8·6
u. ATG‡ (t) – mycolase I – amphotericin (o)	−1, 1(C), 2(A) 3(C), 4(A)	2 × 1·5 mg– 2 × 0·6 μg	All survived*

* Significantly different from a at the 1% level.
† Significantly different from a, f and j at the 1% level. (Logrank χ^2 analysis, Peto and Pike, 1973).
‡ ATG is rabbit anti-mouse thymocyte globulin. 300 μl, injected subcutaneously, was the amount able to prolong the survival of allogeneic skin grafts from 8–10 days to 25–35 days.

Mycolases have been shown to be effective in both immune competent and immunosuppressed animals alone and in combination with existing antimycotic drugs (Davies and Pope, 1978). The latter approach allows reduction in drug dosage and associated toxicity. *In vitro* tests suggest that addition of mycolase gives a 5- to 10-fold reduction in the MIC of a range of antifungal drugs. This is probably due to the increased access to the cell interior through the enzyme-damaged walls.

Mycolases have a wide spectrum of antifungal activity, are of low toxicity and there would seem to be only a remote chance of fungi developing resistance to their attack. The authors believe that mycolases hold considerable promise as an alternative to existing therapy for both systemic and superficial fungal infections.

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same conclusion. Yet one still has to find an explanation for the failure of double half forelimbs to regenerate (ref. 11, S. V. Bryant, B. A. Baca and P. W. Tank, unpublished data). It is conceivable that these paradoxes might be resolved by the consideration of a double gradient system[12], where one gradient corresponds to the antero-posterior axis, the other to the dorso-ventral axis. Such a system provides a simple physiological mechanism for the postulated circumferential gradient of the clockface model, and conforms to the classical embryological work concerning the individual determination of limb bud axes. The ability of a double gradient to explain the above results is currently being investigated.

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Mycolase, a new kind of systemic antimycotic

THERE is a shortage of antimycotic drugs[1,2], especially those for the treatment of systemic fungal diseases. Patients on immunosuppressive therapy or cancer chemotherapy are particularly susceptible to opportunist fungal infections[3], and histoplasmosis, blastomycosis and coccidioidomycosis are major health problems in some tropical countries[4]. For such infections, amphotericin B, 5-fluorocytosine, miconazole and clotrimazole are mainly used. The first of these drugs is nephrotoxic and the second often induces resistant strains of fungi[4]. Griseofulvin is given orally but is effective only for cutaneous infections[4]. A new approach, which we are investigating, is to attack the fungal cell wall by way of the glucans it contains. Preliminary results obtained with 'mycolases', mixtures of enzymes of fungal origin containing various carbohydrases, including chitinase and laminarinase are reported here. We found that, used in conjunction with normally ineffective antimycotic drugs, mycolases were successful against systemic fungal disease in mice.

Most true fungi contain chitin in their cell wall[5–8]; it probably always occurs in the innermost layer but may also be present in outer layers of the surface material as one of various polysaccharides which protect these and other microorganisms[9,10]. Although the pattern and general cell-wall architecture varies among different species, chitin has special importance, as shown by the inability of fungal protoplasts to revert to a viable mycelium without a source of the chitin precursor, *N*-acetyl-D-glucosamine[11].

Chitin has been recognised by other workers as a potential target for therapeutic attack, and specific inhibitors of chitin synthetase have been developed. These compounds, the polyoxins, are unstable *in vivo* and have not been useful clinically[12]. Chitinases occur widely in nature, and although it has been suggested that chitinase might be used to attack and kill chitin-containing microbial pathogens[13], no such effect has been recorded, either *in vitro* or *in vivo*. Because chitin does not occur in any part of the mammalian body structure, chitinase might be expected to be nontoxic to mammals. We have found this to be the case, but chitinase alone is not an effective mycostatic or mycocidal agent (see below).

We have investigated the effect of certain carbohydrases on fungal mycelium by measuring the extent of protoplast formation[14]. Although assays of the enzymatic degradation of isolated mycelial wall components of target pathogens provide useful estimates of activity *in vitro*, the structure and composition of mycelia vary widely between species and are insufficiently understood to relate enzyme levels to *in vivo* activity.

Table 1 Production of protoplasts from vegetative mycelium of *Aspergillus fumigatus*

Enzyme treatment	Protoplast release
Chitinase*	+ §
Laminarinase†	–
Chitinase + laminarinase	+ +
Coprinus extract‡	+ + +

All enzymes were at a final concentration of 240 μg ml^{-1}.
*β-1, 4-*N*-acetyl-D-glucosaminidase (I.C.3.2.1.14, crystalline, Koch-Light).
† β-1,3(4)-glucanase (I.C.3.2.1.6, BDH).
‡ Extract of *Coprinus comatus* (shaggy ink cap), centrifuged, dialysed and lyophilised (mycolase I).
§ +, Protoplast release from hyphal tips only; + +, extensive formation of protoplasts; + + +, complete lysis of mycelium; –, no protoplasts produced.

Table 1 indicates that chitinase alone has no access to its substrate in *Aspergillus*, except near growing tips of the mycelium, where chitinase and chitin synthetase coexist in a state of physiological collaboration to extend the mycelium in normal growth[15]. Laminarinase provides access for the chitinase by degrading surface glucans, so forming an effective combination with chitinase, but alone it is not mycolytic. Natural enzyme mixtures, for example, mycolase I (from *Coprinus*), are much more effective because of the presence of a wider range of carbohydrases. Similar results can be obtained with *Candida albicans* and many other microfungi as target organisms. Enzyme profiles for mycolases will be reported elsewhere.

In vivo assays led to similar conclusions and were carried out by infecting mice by intravenous injection, followed by various treatments with enzymes and/or conventional antimycotic drugs. Results shown in Table 2 are from tests in which BALB/c mice given 5×10^5 spores of *Aspergillus fumigatus* died, if untreated, in 12–14 d. Groups of such infected mice were given intraperitoneal injections of various enzyme preparations, and results are shown for mycolase I (from *Coprinus comatus* fruit bodies) and mycolase III (from *Lycoperdon pyriforme* grown in culture). These were tested with and without amphotericin B or nystatin.

Our conclusions are drawn only from comparisons between groups of mice in which all animals died and other groups in which all survived. In the conditions of the test, maximal sub-toxic doses of the drugs failed to prevent death from fungal infection (Table 2 *i*, *k*). Animals could be saved by enzyme mixtures alone (*d*, *h*) or by the synergistic effect of smaller doses of enzyme and drug (for example, *m*, *n*, *o*, *p*). When mice were infected with *A. fumigatus*

0028–0836/78/0273—0235$01.00

after immunosuppression with rabbit anti-mouse thymocyte globulin (ATG), they could still be treated effectively with enzyme plus drug, indicating that a full immune capacity is not a prerequisite for the effect (*q*, *r*).

Protoplasts of *C. albicans* are more sensitive to amphotericin B than intact cells[17], and we expected that enzymatic damage of the fungal cell walls would facilitate penetration by the existing antimycotic drugs. Although this is certainly true, we have been surprised by the ability of mycolases alone to cure fungal infections.

Acute toxicity tests with mycolases, given intravenously or intraperitoneally, gave an LD_{50} of 15–20 mg per mouse. These data gave a favourable therapeutic index of 150–200. Enzyme preparations fractionated by ethanol precipitation were non-pyrogenic and showed no short-term or longer-term toxic effects in monkeys given a single intravenous injection of 10 mg per kg. Clinical experience will be presented elsewhere.

Mycolases are only poorly immunogenic, and if these enzyme mixtures were used to treat systemic infections in transplant patients or patients receiving cancer chemotherapy, the risks of sensitisation would be minimised by immunosuppression and impaired immunocompetence, respectively. Superficial infection, such as ringworm (*Trichophyton mentagrophytes*) on guinea pig skin, can be treated effectively by topical application of mycolase plus drug; in this kind of situation sensitisation is unlikely to be a problem.

Systemic fungal disease is notoriously difficult to diagnose (most often identified after death) and can take a very rapid course once established. There is a need for effective nontoxic antimycotic agents and we believe that mycolases provide an acceptable treatment for systemic fungal disease.

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Table 2 Fate of BALB/c mice infected systemically with *Aspergillus fumigatus*

	Treatment	Injections (d after challenge)	Dose	Mean survival* (d)
a	None (controls)	—	—	13.5
b	Chitinase + laminarinase	1,2,3	3 × 1.5 mg	26.3
c	*Coprinus* (crude)	1	1.5 mg	21.0
d	*Coprinus* (crude)	1,2,3	3 × 1.5 mg	All survived*
e	*Coprinus* (crude)	12	4.5 mg	35.6
f	*Lycoperdon* (crude)	1	1.5 mg	11.2
g	*Lycoperdon* (purified)†	1	100 µg	15.8
h	*Lycoperdon* (purified)	1,2,3	3 × 100 µg	All survived*
i	Amphotericin B	1	5.0 µg	23.2
j	Amphotericin B	1	0.6 µg	16.0
k	Nystatin	1	100 µg	30.6
l	Nystatin	1	12.5 µg	14.6
m	*Coprinus* plus amphotericin (*c* + *j*)	1–2	1.5 mg + 0.6 µg	All survived*
n	*Coprinus* plus nystatin (*c* + *l*)	1–2	1.5 mg + 12.5 µg	All survived*
o	*Lycoperdon* plus amphotericin (*f* + *j*)	1	1.5 mg + 0.6 µg	All survived*
p	*Lycoperdon* (purified) plus amphotericin (*g* + *j*)	1–2	100 µg + 0.6 µg	All survived¶
q	ATG before challenge‡	1	300 µl	8.6
r	ATG (*q*), plus *Coprinus* plus amphotericin (*m*)	1 (ATG) 1–4	2 × 1.5 mg 2 × 0.6 µg§	All survived*

Groups of mice, usually 5 but in some cases 10, weighing between 22 and 25 g, were challenged by intravenous injection with 5×10^6 spores of *A. fumigatus*. Control (untreated) mice usually died after 12–14 d (*a*); the time was 8.6 d for control mice in the case of *h*. Enzymes and drugs were used as described in Table 1. The toxic doses of amphotericin B and nystatin were approximately 10 µg and 200 µg, respectively. Treatment was administered intraperitoneally.

* Significantly different from *a* at 1% level.

† *Lycoperdon* (*L. pyriforme*) was grown in culture and the culture supernatant fluid used untreated ('crude') or fractionated by gel filtration (the retarded fraction of Sephadex G200 columns was used), and referred to as 'purified'.

‡ ATG is rabbit anti-mouse thymocyte globulin; this was injected subcutaneously and the amount used (300 µl) was that able to prolong the survival of allogeneic skin grafts from 8–10 d to 25–35 d.

§ In this test ATG was injected the day before challenge at the same dose as in *q*. Mycolase I was given on days 1 and 3 (dose 1.5 mg) and drug on days 2 and 4 (dose 0.6 µg) after challenge.

¶ Significantly different from *a*, *f* and *j* at 1% level (Log rank χ^2 analysis[16]).

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Inherited disorders in the regulation of serum calcium in rats raised from parathyroidectomised mothers

In a series of experiments to study the influence of maternal endocrine dysfunction on the embryonic development in rats, we have found that the first generation (F_1) rats born to parathyroidectomised (Px) mothers possess a slightly, but significantly, lower serum calcium level than in normal control rats[1]. Furthermore, the serum calcium level in the rats of the third or fourth generations (F_3 or F_4) developed by brother–sister mating was still at a subnormal level. To characterise calcium metabolism in these animals, the response of serum calcium to removal of the parathyroid gland was examined in the F_1, F_3 and F_4 rats. We report here that F_3 and F_4 rats raised from mothers parathyroidectomised on the 5th day of pregnancy showed a less marked decline in serum calcium level following parathyroidectomy as shown in the first generation, suggesting that the functional alteration resulted from the intrauterine hypocalcaemia persists in subsequent generations.

Female rats of highly inbred Wistar–Imamichi strain, weighing 250–300 g, were obtained from the Animal Breeding Research Laboratory (Ohmiya) and were mated. The day on which sperm were present in the vaginal smears was taken as day 0 of pregnancy. The surgical parathyroidectomy was carried out on the 5th day of pregnancy under ether anaesthesia. After the operation, mother rats were given 1% calcium chloride solution as drinking water for 3 d before parturition and for the first 3 d of lactation. Without calcium chloride solution supplementation, they died of tetanic convulsions on the 21st or 22nd day of pregnancy. Control pregnant rats were sham operated. Adult female rats at the F_1 and at the F_3 or F_4, weighing 250–280 g, were parathyroidectomised unilaterally or bilaterally under ether anaesthesia. All animals used were maintained in an air-conditioned room (22 ± 2 °C, 55 ± 5%) with a lighting schedule of 14 h light (0600–2000) and 10 h

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